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This study involves measurement of neutrophil migration to FMLP, adherence, and oxidative burst capacity in medical students as a result of their participation in simulated field medical operations. Two studies of samples of medical students before, during, and after the exercise have been completed, and findings from these studies suggest that the experience of the exercise was associated with decreases in neutrophil adherence but did not produce any changes in cell migration to a chemoattractant or in ability to undergo oxidative bursts. However, several logistical and assay problems arose during these studies; though these studies allowed us to resolve these problems, the data from the first two studies must be viewed cautiously. Replication of this effect in subsequent studies planned for the coming year will be necessary to drawn firmer conclusions.

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I. Introduction

The effects of stress on immune system status and function have been of interest for many years, but it is recently that this topic has received a great deal of systematic research attention. Studies of acute and chronic stress with animal and human subjects have suggested that stress can affect immune system functioning by reducing cells' ability to proliferate when stimulated by a mitogen, increasing the likelihood of reactivation of latent viruses, and, in some cases, by affecting the numbers of leukocytes and various subpopulations of lymphocytes (cf Kiecolt-Glaser & Glaser, 1987). Though as a whole unsystematized, behavioral immunology has emerged as a rapidly developing field of scientific inquiry directed, in part, at better understanding the relationships between psychological and physiological events associated with stress on the one hand and immune function and health on the other.

During the past decade, research has clearly established links among the nervous, endocrine, and immune systems as well as demonstrating psychological influences on immune function and status. Research has indicated that immune responses can be conditioned (e.g., Ader, 1981; Ader & Cohen, 1975) and several studies have shown that stress affects the strength of immune response to a variety of stimuli (cf. Kiecolt-Glaser & Glaser, 1987). Studies have chiefly used lymphocyte proliferation measures in this work, counting mitogen-induced replicates of T or B cells and relating proliferative ability to stress or other conditions. Other measures have been used, including counts of lymphocyte subpopulations, natural killer cell activity (lysis of target cells), quantitation of antibody titers to latent viruses, tests of delayed hypersensitivity, and tumor rejection. Choice of measures in these studies is based on several factors, and has not led to uniform study of all aspects of immune function; instead, some aspects of immune response have been more fully studied than others. A relatively neglected area is the effect of stress on neutrophil, or polymorphonuclear granulocyte (PMN) activity and function.

One study examined the effects of a stressful 77-hour sleepless vigil on phagocytic capacity of neutrophils. Rate of phagocytosis was lower during the vigil than before or after it, but some evidence of adaptation by the end of the vigil was also reported. Plasma cortisol and urinary catecholamines appeared to be negatively correlated with phagocytosis. A second study (Palmblad, Petrini, Wasserman, & Akerstadt, 1970) measured PMN adherence before and immediately after a 48-hour sleepless period and five days afterward. They found no evidence of significant changes in adherence. However, measures in this study were taken before and after the vigil: no intermediate measures during the sleepless period were reported. It is therefore possible that observations of meaningful changes was obscured by adaptation towards the end of the session.

Neither of these studies can be considered conclusive regarding the effects of stress or sleep deprivation on neutrophil activity. Sample sizes were small, little information about the subjects was reported,

and the difficulty in separating the effects of stressful tasks and of sleep deprivation cloud interpretation of the findings.

As the field evolves it is necessary to examine aspects of immunity not commonly studied and stressors that more accurately reflect stressors that might be experienced in real-world settings. Our approach has been to study the neutrophil, a basic, non-specific form of immune defense against bacterial incursion, and to examine the effects on this cell of a week-long Bushmaster exercise, a field operation in which military medical students are involved in a simulation of MASH operations. The exercise combines several forms of stress common in actual operations, including sleep deprivation, physical exercise, threat, uncomfortable living conditions, and evaluation apprehension. Clearly, it will not be possible to separate out the effects of these various stressor conditions so that we can determine those that have effects on neutrophil function. However, the naturalistic aspects of the exercise and the control afforded both in terms of conditions such as nutrition and of experiences during the exercise provide the opportunity of studying a real-world event under relatively well-controlled conditions. The Bushmaster exercise is held three times each year; during year one, we studied two of these sessions.

II. Progress Report

A. Research objectives: The primary goal of our research is to investigate the effects of stress during and after a military medical field exercise on neutrophil functions, including cells' ability to migrate, adhere, and undergo respiratory bursts. The relationships among adrenal hormones, stress, and neutrophil function will also be investigated. The exercise, called Bushmaster, is required of all fourth year medical students at USUHS and is held three times each year. Because of limits on our ability to assay large numbers of samples of fresh blood, and because each exercise session may differ slightly, our strategy is to assess stress and neutrophil activity from several Bushmaster sessions and combine the data across a two-year period.

B. Procedures and results: Since January, 1988, when ONR funding was completed, we have collected data from two sessions held near San Antonio, Texas. Our first assessment was a pilot study, designed to test the feasibility of procedures adopted due to logistic difficulties in collecting data. A total of five medical students and four control subjects participated, and we were able to collect pre-exercise, during-exercise, and post-exercise samples (upon returning to campus) on five of the medical student subjects. Controls provided samples once, at the time of the pre-exercise assessment for Bushmaster participants. The pilot was successful in establishing logistical arrangements and procedures for subsequent sessions, and findings from these subjects were of some interest.

Neutrophils were evaluated for random migration and chemotaxis using N-formyl-methionyl-leuccyl-phenylalanine (FMLP) as a stimulant at 1×10^{-7} M in a 48 well modified Boyden chamber. The chamber was set up with the lower portion of the chamber containing either FMLP (chemotaxis) or culture media (random migration). A filter membrane

with 5.0 um size pores was placed over the lower portion of the chamber and neutrophils at 2 x 10^6 were placed in the upper wells of the chamber. Cells were allowed to migrate for one hour at 37° C after which the filter membrane was removed and stained before evaluating for numbers of cells migrating toward the chemoattractant using an Optimax image analyzer.

Neutrophil adherence was determined by adding 1 x 10^6 cells/ml to a glass surface and incubating in a 37° C humidified atmosphere for 15 minutes. After incubation the adherent cells were washed and stained with Wright's stain. The number of adherent cells were determined using an Optimax image analyzer.

Results of these neutrophil studies indicated first that the total white blood cell count for Bushmasher participants was slightly elevated (X = 13.3 x 106) immediately following the exercise as compared with pre-exercise levels (X = 11.8 x 106), but neutrophils isolated from the blood of this small sample of individuals were found to have a decreased ability tc adhere after exercise. The ability of cells to migrate to a chemoattractant (FMLP) was not markedly different from neutrophil migration of control subjects. However, there were increases in both random and stimulated migration after the exercise (X = 2984 random, X = 6436 stimulated) from pre-exercise levels (X = 2256 random, X = 5263 stimulated). There were also no differences in the ability of "pre and post-exercise" cells to undergo oxidative bursts. Migration and NBT assays could not be performed on cells collected during the session. However, the ability of the cells to adhere was markedly reduced in "stressed" samples; post exercise numbers of adherent cells (X = 163) were substantially lower than they had been before the exercise (X = 2908).

The finding that cells exhibited decreased ability to adhere following the exercise is important because the overall function of neutrophils is dependent on their ability to adhere and provides a possible explanation for an earlier finding that chronic stress is associated with increased numbers of neutrophils in circulation. In a recent study, we performed WBCs (with differential) on blood samples collected from a small group of people living near the damaged Three Mile Island nuclear station and from control site 80 miles away (McKinnon et al, in press). The findings indicated that the TMI area subjects had higher numbers of circulating neutrophils than did control subjects. Further, elevated levels of epinephrine were positively correlated with numbers of neutrophils.

The second Bushmaster sample consisted of six Bushmaster participants and three normal control subjects. Samples were collected from Bushmaster participants and controls twice before the exercise, at one-week intervals and once immediately after returning to campus following the exercise. Due to illness, however, data were available for only one control subject post-exercise. Parameters evaluated for the second Bushmaster exercise included determination of total white blood cell numbers and neutrophil adherence, migration and oxidative burst potential of isolated cells. The results of these studies are presented in Tables 1-3.

Total WBCs found in the blood of Bushmaster subjects were elevated both before and after the exercise compared to control subjects (Table 2), although cell numbers were within normal ranges at both study times evaluated. The total number of neutrophils in the blood were also slightly higher after the exercise compared to controls and were within normal limits.

Neutrophils isolated from the blood of Bushmaster subjects participating in this second exercise again showed decreased capability to adhere to a glass surface when evaluated after the field exercise as compared to neutrophils from these same subjects tested prior to the exercise. Similarly, Bushmaster neutrophil adherent characteristics were less than seen with neutrophils from normal control subjects (Table 1), although large variations in individual adherent responses in this limited group of study subjects prevents any meaningful statistical interpretation.

Results of neutrophil migration studies are presented in Table 2. Neutrophils from Bushmaster participants had a decreased ability to migrate randomly in samples tested immediately after the exercise compared to prior to participation and compared to control subjects. In contrast, chemotactic migration toward FMLP by Bushmaster neutrophils was not different than was seen when tested prior to exercise and compared to migration characteristics of control subjects. As with adherence studies, the limited number of study subjects evaluated to date prevent a meaningful statistical evaluation of the migration data.

Studies of neutrophil oxidative burst potential as measured in nitroblue tetrazolium (NBT) reactions are presented in Table 3. Unstimulated and FMLP stimulated neutrophils from Bushmaster participants had NBT reactions which were comparable at pre-exercise and post-exercise evaluation times and were similar to normal control neutrophil reactions tested at the same time points.

Neutrophil response data collected from the first (pilot) and second Bushmaster exercise were comparable and, as expected, refinement in logistics and blood handling procedures that were implemented in the second study provided greater clarity of results as the study progressed. Taken together, it appears in these early studies that the decrease in ability of unstimulated cells to adhere to glass surfaces is correlated with a decrease in the ability of the cells to migrate randomly, at least as seen in Bushmaster study 2. In contrast, the cells appear to have a normal ability to migrate when stimulated with FMLP and a normal capability to undergo an oxidative burst reaction before and after the exercise. Additional studies of Bushmaster subjects will help delineate the relationships between migration and adherent characteristics. Definitive studies which might clarify this question would include evaluation of the presence of the adherent proteins MO-1 on the surface of neutrophils from Bushmaster participants and the expression of these molecules following myeloperoxidase degranulation, a process that involves an oxidative reaction.

Hormonal assays on samples from the Bushmaster subjects have not been completed, but when they are, the results may suggest how the

changes observed in neutrophil activity occurred. Of substantial interest is the finding that stress may increase the number of circulating neutrophils but after certain of their functional responses as measured in initial assays. We are currently involved in studying the next Bushmaster exercise, with methods and assays developed in the first year.

The overall function of neutrophils, including the ability to adhere, migrate and undergo an oxidative burst reaction is important to understanding the relationship between these activities and the overall physiological status of the study subject and may provide an explanation for an earlier finding that chronic stress is associated with increased numbers of circulating neutrophils.

Further studies evaluating clinical outcomes in subjects exhibiting stress-induced changes in PMNs are needed in order to determine whether these changes have any significance for health. Clearly, both studies completed during Year 1 should be considered as pilot studies, and we are currently involved in studying the next Bushmaster exercise, with methods and assays perfected during the first year.

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Table 1

Mean Cell Numbers before and after Bushmaster Exercise, Pilot II

	Pre-Exercise	Post-Exercise	Control
Total WBC (number x 10^6)	4.48	4.28	3.96
Total PMN (number x 10^6)	2.01	2.18	2.02

Table 2

Mean (standard deviation) neutrophil migration and adherence values pre- and post-Bushmaster (two pre-samples were comparable and were averaged) and for normal controls.

	Bushmaster participants pre-exercise	Bushmaster participants post-exercise	Normal control
Random Migration	43.5 ± 29.9	21 ± 9.6	39.5 ± 2.5
Stimulated Migration	181.5 ± 30.4	161.3 ± 24.1	157.7 ± 49.4
Adherence	1216.8 ± 232.8	945.0 ± 56.2	1501.5 ± 68

Table 2

Oxidative Burst Reactions of Resting and Stimulated Neutrophils from Bushmaster Participants and Control Subjects

	Bushmaster Participants		Control
	Pre-Exercise	Post-Exercise	Subjects
Stimulant	(Mean Score±SEM)		
None	160.0 ± 10.1	153.0 ± 18.2	181.0 ± 6.4
FMLP 1×10^{-7}M	362.3 ± 20.7	384.3 ± 50.8	415.5 ± 51.3

*Mean Score--200 neutrophils were graded for NBT reaction based on an intensity reaction ranging from 0 (no reaction) to 4+ (majority of cell containing NBT reaction). The number of cells with each score were determined and the total score for the 200 cells were calculated.

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